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A high-efficiency, low-toxicity, phospholipids-based phase separation gel for long-term delivery of peptides

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ABSTRACT

Peptide and protein drugs are currently under rapid development attributed to their high potency and efficacy in therapy. Their successful delivery, however, is highly limited by their short half-life, fast degradation and rapid clearance. Here, we present a high content phospholipids-based phase separation gel (PPSG), which is readily injectable due to its low initial viscosity and can rapidly transform into an in situ implant after injection upon exposure to an aqueous environment. A selected model peptide, octreotide acetate, is loaded into PPSG and achieves sustained release profiles for one month in rats. In addition, the local irritation caused by ethanol contained in PPSG is ethanol content-dependent and the irritation of PPSG with 70% phospholipids content can be eliminated by partially replacing ethanol with medium chain triglyceride. The mechanisms underlying phase transition of PPSG are based on water-insolubility of phospholipids. Our findings demonstrate that PPSG is a readily injectable, highly safe and efficient in situ forming implant for sustained delivery of peptides.

1. Introduction

During the past decades, biopharmaceuticals have attracted much attention in drug discovery. Since the successful entry of the first recombinant protein onto the market in 1980s, FDA has approved above 400 biopharmaceuticals [1]. Nowadays, biopharmaceuticals are becoming the leading therapeutics owing to their clinical and commercial success [2]. It is reported that worldwide sales of all biologics in 2010 was around US$100 billion mark [3], and it is expected that more than 50% of new drug approvals will be biologics [4]. Peptides and proteins are of a large class of biopharmaceuticals under investigation. However, their successful delivery is limited by their short half-life. In order to improve their pharmacokinetic profile, various formulations including nano-/micro-particles [5,6], hydrogels [7,8] and scaffolds [9,10] are developed. Although progresses have been made, the long-term release of peptides and proteins is still a big challenge because of the hardly controlled initial burst release, low compliance or safety issues. Therefore, an ideal delivery system should be safe, patient compliable, and able to provide a stable and effective sustained release profile with desired initial burst release.

In situ forming implant is a promising system due to its injectability and the ability of forming drug depots [11–13]. However, its practical use is highly limited by certain drawbacks including high viscosity [14–16], additives and organic solvents induced toxicity [17–20], and relatively fast drug release [21–23]. Among those commonly investigated implants, phospholipids-based organogel is an attractive one because of the non-toxicity, biocompatibility and ready availability of phospholipids [24–26], but it is also suffered from high viscosity induced by the growth and overlap of reverse tubular micelles [27,28]. It is recommended that phospholipids concentration in organogel should be lower than 15% to reduce viscosity efficiently [13,24], while lower phospholipids content will in turn make drug release much faster.

In this present work, we report, for the first time, a novel phospholipids-based phase separation gel (PPSG) that contains as high as 70% (w/w) phospholipids but has a very low initial viscosity. It is reported that the commonly used organic solvents in phospholipids organogel include aliphatic ester, alkane, cyclane and

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amine [27]. As we know, phospholipids are also soluble in ethanol while almost insoluble in aqueous solution. Thus, the phospholipids dissolved in ethanol will be solidified and become a drug depot when exposed to aqueous solution. Herein, we aim to utilize this property to develop a readily injectable in situ forming implant (PPSG), test its phase transition properties in vitro and in vivo, evaluate its toxicity in rabbit, discuss the mechanisms of phase transition and eliminated toxicity and investigate its ability for sustained delivery of peptides or proteins using octreotide acetate (OCT) as a model peptide.

2. Materials and methods

2.1. Materials

Phospholipid (E80) was purchased from Lipoid (Germany). Medium chain triglyceride (MCT) was provided by Beiya Medical Oil Co. Ltd (Tieling, China). Octreotide acetate was offered by Chengdu Kaijie Bio-Pharmaceutical Co. Ltd. (Chengdu, China). Acetonitrile (HPLC grade), methanol (HPLC grade) and Fehling's solution were purchased from Kemiou (Tianjin, China). All other agents were of analytical grade or better.

2.2. Animals

The healthy male Sprague–Dawley rats (200–260 g) and Japanese big-ear rabbits (2–3 kg) were purchased from Laboratory Animal Centre of Sichuan University (Chengdu, China). All the animal experiments were approved by the Institutional Animal Care and Ethic Committee of Sichuan University. The animals were housed in cages (5 rats per cage and 1 rabbit per cage) with free access to food and water. Prior to use they were acclimatized for at least 7 days.

2.3. Preparation of phospholipids-based phase separation gel (PPSG)

The PPSGs without MCT was simply prepared by dissolving different amount of E80 in absolute ethanol under magnetic stirring at room temperature. When preparing the PPSGs with MCT, 15% (w/w) of ethanol was replaced by MCT. The OCT-loaded PPSG was prepared by dissolving E80 and OCT in 85% (v/v) ethanol that contained 15% aqueous buffer (pH 4.0) to maintain the stability of OCT.

The PPSGs containing 40%, 50%, 60% and 70% (w/w) E80 were prepared and presented as PPSG-40, PPSG-50, PPSG-60 and PPSG-70, respectively. PPSG with or without MCT is presented as PPSG-(+) or PPSG-(−). The composition of various PPSGs was listed in Table 1.

2.4. Viscosity measurement

The viscosity of PPSGs was measured using a digital viscometer (DV-C, Brookfield Engineering Laboratories, Inc., USA). Briefly, a certain volume of PPSG was added to a container, which matched with the used rotator in size. The added PPSG should submerge the rotator. Before measurement, the rotating speed was set up to ensure the torque value in the range of 10–100%. The viscosity value was not recorded until all the parameters shown in the screen became stable.

2.5. Effects of water content on the viscosity of PPSG

The PPSGs were mixed with different amount of PBS (0.01 M, pH 7.4) by stirring for at least 10 min at room temperature. The PBS content in the resultant mixture was in the range of 0–50% (w/w). The viscosity of the resultant homogeneous mixture was measured as above. The effect was presented as viscosity-PBS content curve.

2.6. Ternary phase diagram of PPSG

A series of E80 solutions in ethanol were prepared, to which aqueous solution was added drop by drop under magnetic stirring. The added amount of aqueous solution was recorded when the mixture became turbid. The ternary phase diagram was drawn based on the amount of aqueous phase, ethanol and E80.

Table 1
Composition of PPSGs.

Gels	Composition (%, w/w)		
	E80	Ethanol	MCT
PPSG-40-(+)	40	45	15
PPSG-40-(−)	40	60	0
PPSG-50-(+)	50	35	15
PPSG-50-(−)	50	50	0
PPSG-60-(+)	60	25	15
PPSG-60-(−)	60	40	0
PPSG-70-(+)	70	15	15
PPSG-70-(−)	70	30	0

2.7. In vitro release

Approximately 0.2 g OCT-loaded PPSG-40 or PPSG-70 (containing 5 mg/g of OCT) and 1 ml OCT solution (containing 1 mg/ml of OCT) were added to the dialysis bags (molecular weight cut-off was 8–14 kD), respectively. After tightly bundling the two ends, the sample loaded dialysis bag was soaked in 4 ml release medium (PBS or ethanol contained PBS, pH 7.4), followed by shaking in a horizontal shaker at 100 rpm and 37 °C. At predetermined time intervals, the medium outside the dialysis bag was collected and replaced with 4 ml fresh medium. The collected medium was diluted to 5 ml and OCT content was determined by HPLC (Agilent 1260 infinity, USA).

2.8. Microscopy

A thin layer of PPSGs was spread on a clean glass slide. After air-drying for one day, the micro-structure of PPSGs was observed under a microscope (Olympus IX71, Japan).

2.9. Solvent diffusion

A) Water diffusion: Approximately 0.4 g PPSG-40 or PPSG-70 was added to dialysis bags (molecular weight cut-off was 8–14 kD). After tightly bundling the two ends, the dialysis bags were immersed into 200 ml PBS (pH 7.4), followed by magnetic stirring at room temperature. At fixed time intervals, the dialysis bags were taken out and the loaded PPSGs were collected. The weight of the collected PPSGs was recorded and approximately 2-fold methanol (v/w) was added to dissolve PPSGs. The water content in the collected PPSGs was determined using Karl Fischer method (Mettler Toledo V20 Volumetric KF Titrator, Germany).
B) Ethanol diffusion: Similarly, ~0.4 g PPSG-40 or PPSG-70 was added to dialysis bags and immersed into 200 ml PBS (pH 7.4) under magnetic stirring. At fixed time intervals, 4 ml PBS was collected and replaced with 4 ml fresh PBS. The ethanol content in the collected PBS was determined using HPGC (Agilent GC7890A, USA).

2.10. Dermal irritation in rabbits

The rabbits were depilated (3 × 3 cm) at two backsides 24 h before subcutaneous injection of various formulations. The rabbits were divided into three groups with 2 rabbits in each group. The rabbits in group 1 were injected at two sides with physical saline and PPSG-70-(+), respectively; group 2 were injected with PPSG-40-(+) and PPSG-70-(+); group 3 were injected with PPSG-70-(−) and PPSG-70-(+). At 4 or 14 d post-injection, the skin at injection site of each rabbit was visually inspected and collected for pathological section, HE staining and microscopy.

2.11. Pharmacokinetics in rats

The rats were divided into 4 groups (5 rats for each) and fasted for 12 h before subcutaneous injection of OCT solution, OCT-loaded PPSG-70-(+), commercially available OCT microspheres (Sandostatin LAR, Novartis Pharma Schweiz AG, Switzerland) at the dose of 20 mg/kg. In addition, LAR was also injected intramuscularly according to the product instructions. At fixed time intervals, 300 μl of blood were collected into heparinized tubes, followed by centrifugation (4500 rpm, 10 min) to separate plasma. An aliquot of 100 μl plasma was collected and stored at −20 °C for further analysis. The OCT content in plasma was quantified by LC-MS/MS (Agilent 1200 HPLC/Agilent 6410B MS).

2.12. Data and statistical analysis

The data are presented as mean ± s.d. (standard deviation). Statistical analysis was performed using two-tailed Student's *t*-test.

3. Results and discussion

3.1. Formation of low-viscosity PPSGs

In this work, the injectable PPSGs containing various concentrations of E80 were prepared. As shown in Fig. 1a, PPSGs before phase transition are clear and transparent solutions in appearance, suggesting that E80 is well dissolved. In addition, the color becomes darker when E80 content gets higher, but addition of medium chain triglyceride (MCT) has no effect on the appearance (appearance of all PPSGs solutions is shown in Supplementary Data Fig. S1). The